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# Greedy reduction of *Bacillus subtilis* genome yields emergent phenotypes of high resistance to a DNA damaging agent and low evolvability

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## HIGHLIGHTS

<b>Delivery:</b> Library of 298 strains with genomes reduced to as small as 1.48 Mb in size. <b>Method:</b> Deletions were accumulated through a two-step deletion cycle.	<b>Characteristics:</b> Growth was progressively impaired with genome reduction. Accumulation of synthetic-sick interactions. Transcriptional signatures of a constitutive thiol-induced oxidative stress. Decreased frequency of spontaneous mutation (up to 100-fold).
<b>Conclusion:</b> Synthetic inactivation of the mismatch repair system resulted in an increased mutation rate and enabled the selection of competitive genome-reduced isolates. This establishes an engineering framework for efficient genome streamlining of chassis strains for synthetic biology applications.	

## INTRODUCTION

We investigate the consequences of genome reduction in *Bacillus subtilis*, which has many advantages for genome engineering including natural competence, efficient chromosomal replacement, existing repertoires of non-essential regions and genes<sup>1</sup>, and availability of libraries of parts for fine-tuning gene circuit expression<sup>2</sup>. As such, *B. subtilis* is an ideal candidate for the development of a well-characterized minimal chassis in which complex metabolic and regulatory circuits can be implemented and fine-tuned. First, we applied a greedy genome reduction strategy in which deletions in large dispensable regions were serially combined starting from the largest. At each step, the fittest clonal population was used for the next deletion cycle. This strategy generated a synthetic evolutionary tree of 298 strains with reduced genomes, the smallest genome (of 2.73 Mb) having lost 1.48 Mb in size<sup>3</sup>.

## GREEDY REDUCTION OF THE *B. subtilis* GENOME

- We built a library of 146 genome-reduced strains, each with a single deletion of a dispensable chromosome region, collectively covering ~76% of the *B. subtilis* 168 genome<sup>1</sup>. We selected the 48 largest dispensable regions (from ~131 kb down to ~16 kb), covering 57% of the genome, for stepwise deletion (**Figure 1**).
  - Deletions were accumulated through a two-step cycle (**Figure 2**):
    - (i) Deletion of a chromosomal region by homologous replacement with a selectable cassette.
    - (ii) Eviction of the cassette via spontaneous pop-out recombination to generate marker-free deletions, preparing genome-reduced strains for further cycles.
  - From MGP052<sup>p/o</sup> (p/o for pop out) two separate lineages were pursued accumulating 10 and 17 deletions, respectively.
- Finally, we obtained MGP184 (~2.87 Mb) and MGP254 (~2.73 Mb), two strains respectively reduced by ~1.34 and ~1.48 Mb relative to the *B. subtilis* 168 strain.

## LOSS OF FUNCTION AFFECTS FITNESS OF GENOME-REDUCED STRAINS

- Over 272 strains carrying a single deletion of dispensable regions<sup>1</sup> (**Figure 3**):
  - ~ 24% showed increased growth rates, while ~ 40% exhibited decreased growth rates compared to the Master strain (MS).
  - ~1% of these strains displayed significantly increased biomass production, whereas 72% exhibited significantly decreased biomass production, possibly due to challenges in carbon source consumption or increased cell maintenance.
- Cell fitness decreased with the accumulation of deletions in the genome.
- Accumulation of deletions globally led in cumulated synthetic-sick interactions within each lineage.
- When comparing MGP181 and MGP254 with MS (using a fold change cut-off of 1.5), ~ 83% and 72% of core genome genes (present in all genome-reduced strains) showed no significant differential expression.

These findings highlight the transcriptional network's robustness against extensive genome reduction and suggest that synthetic-sick interactions may stem from deregulation of only a few genes.

- Transcriptome analysis revealed a common feature in our genome-reduced strains: **upregulation of genes involved in cell responses to oxidative stresses**.

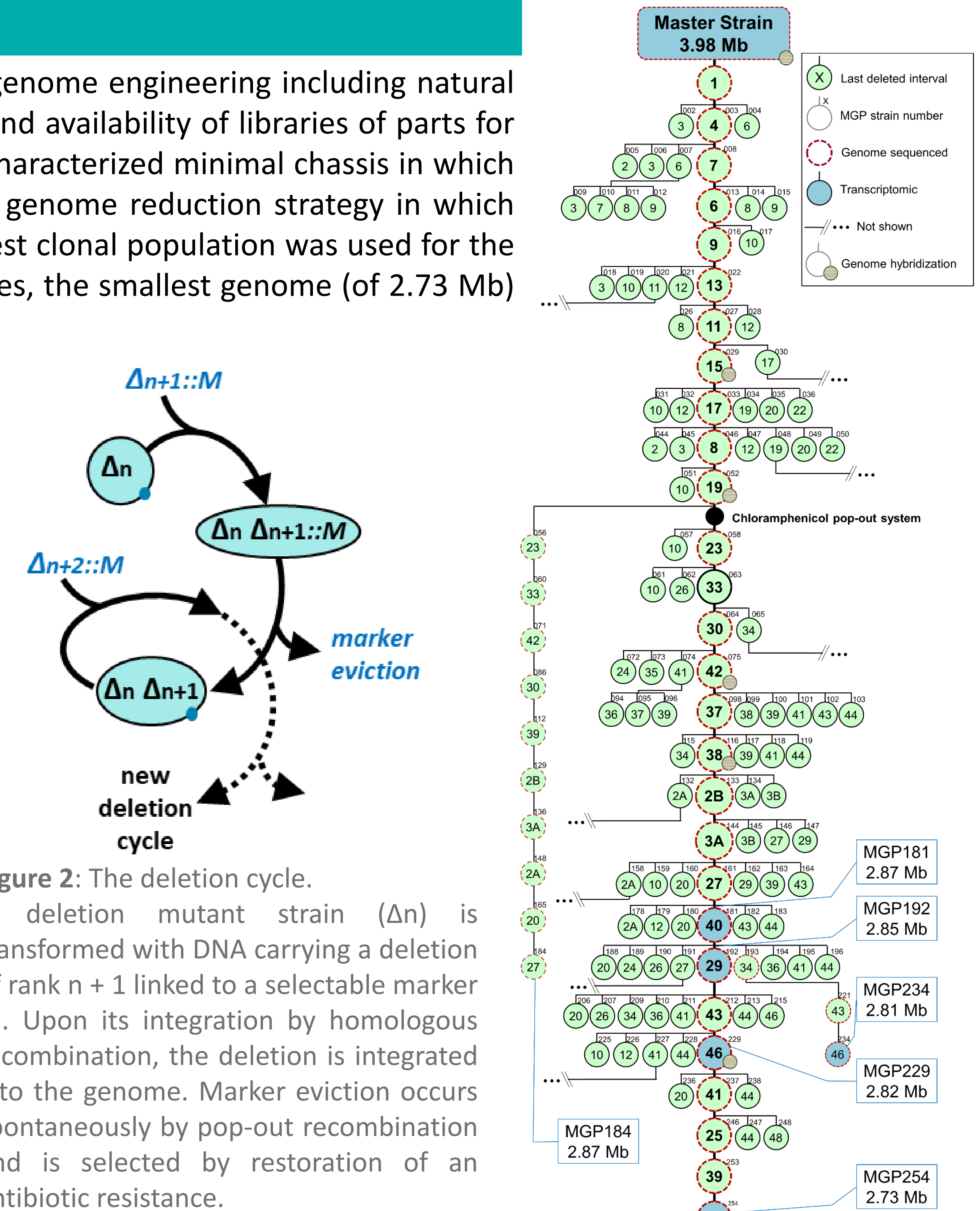
## GENOME-REDUCED STRAINS EXHIBIT LOW SPONTANEOUS MUTAGENESIS

- Low mutation frequency in MGP229<sup>p/o</sup> (**Table 1**) relies entirely on a functional mismatch repair (MMR) system.
- The MMR system might be more efficient in genome-reduced strains, drastically reducing spontaneous mutagenesis and likely limiting evolution.

Genome-reduced strains have limited potential for short-term adaptive evolution.

## CONCLUSION

Design and construction of efficient cell factories shall be envisioned as an engineering framework coupling reductive and evolutive cycles with high-throughput screening. Reductive cycles encompass successive deletions of unwanted genomic regions in a context of low evolvability. Evolutive cycles include a condition-dependent increase of spontaneous mutagenesis for experimental evolution or the integration in a chromosomal landing-pad of a library of DNA fragments from the *Bacillus* species pangenome. This blueprint will allow selecting efficient cell factories at each cycle of deletion, before entering the next cycle and pave the way for innovative construction of synthetic chassis.



**Figure 1:** A synthetic evolutionary tree of 298 strains with genomes reduced by up to 1.48 Mb.

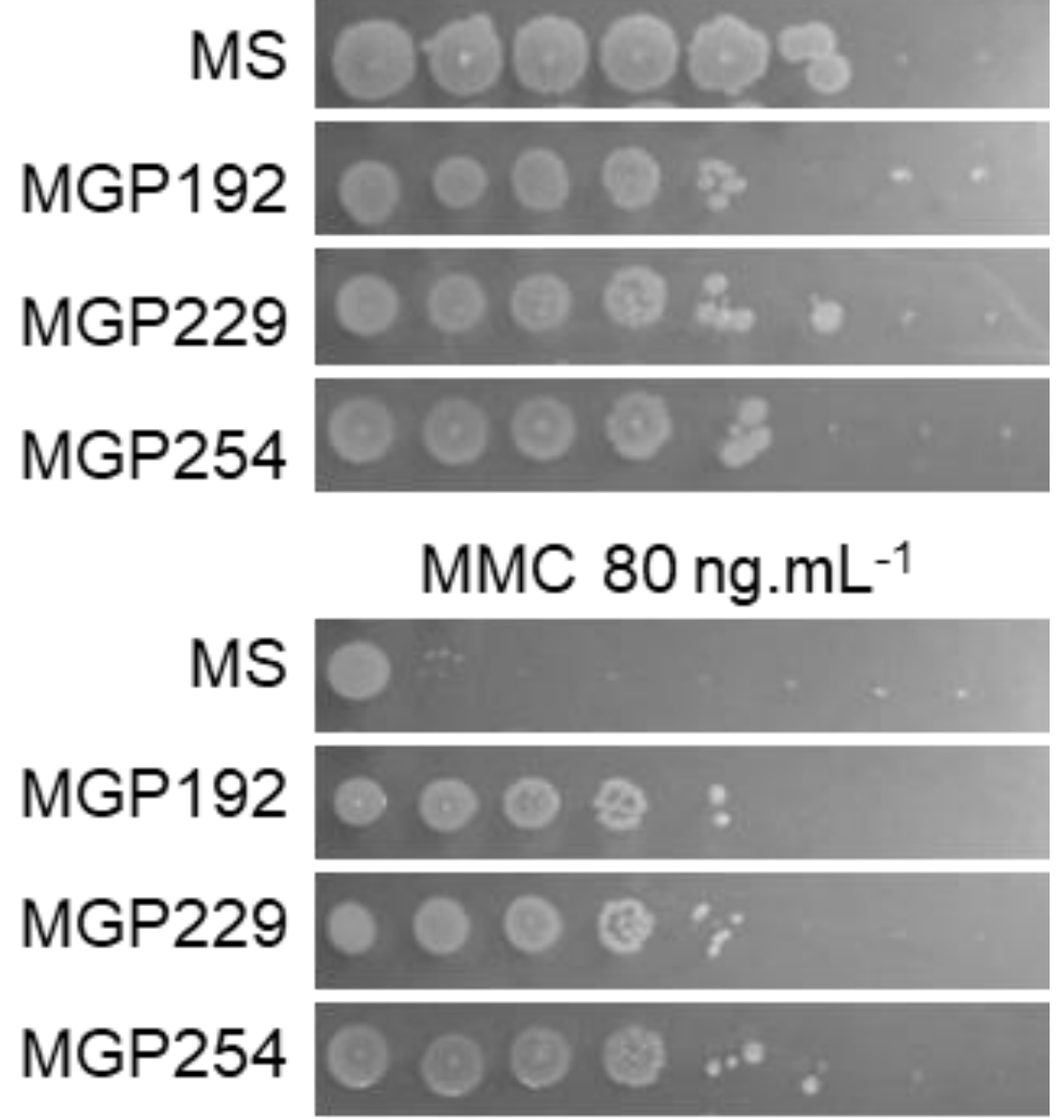
**Figure 2:** The deletion cycle. A deletion mutant strain ( $\Delta_n$ ) is transformed with DNA carrying a deletion of rank  $n + 1$  linked to a selectable marker M. Upon its integration by homologous recombination, the deletion is integrated into the genome. Marker eviction occurs spontaneously by pop-out recombination and is selected by restoration of an antibiotic resistance.

## GENOME-REDUCED STRAINS ARE HIGHLY RESISTANT TO THE DNA DAMAGING MITOMYCIN C

The DNA-damaging agent mitomycin C (MMC) induces double-stranded DNA breaks.

LexA, which controls the SOS response to DNA damage, is constitutively active in genome-reduced strains.

The upregulation of the LexA regulon may contribute to the higher MMC resistance (**Figure 4**).



**Figure 4:** Genome-reduced strain sensitivity to MMC. Cultures were plated on LB agar plates with either 0 or 80 ng/mL MMC and incubated at 37°C for 72 hours.

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